

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Overexpression of the muscle-specific protein, melusin, protects from cardiac ischemia/reperfusion injury.

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/146596> since 2021-09-29T01:24:02Z

Published version:

DOI:10.1007/s00395-014-0418-9

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

The final publication is available at Springer via <http://dx.doi.org/10.1007/s00395-014-0418-9>

Penna C, Brancaccio M, Tullio F, Rubinetto C, Perrelli MG, Angotti C, Pagliaro P, Tarone G.
Overexpression of the muscle-specific protein, melusin, protects from cardiac
ischemia/reperfusion injury. Basic Res Cardiol. 2014 Jul;109(4):418.

Overexpression of the Muscle Specific Protein, Melusin, Protects from Cardiac Ischemia/Reperfusion Injury

Claudia Penna^{*1,2}, Mara Brancaccio^{*3,4}, Francesca Tullio^{1,2}, Cristina Rubinetto^{3,4}, Maria-Giulia Perrelli^{1,2},
Carmelina Angotti^{1,2}, Pasquale Pagliaro^{*1,2}, Guido Tarone^{*3,4}

¹Dept of Clinical and Biological Sciences, University of Torino, Italy; ²National Institute for Cardiovascular Research, Bologna, Italy and ³Dept of Molecular Biotechnology and Health Science, ⁴Molecular Biotechnology Center, University of Torino, Italy.

* These authors contributed equally to this work

Short title: Cardioprotection by melusin

Address for the correspondence:

Dr Pasquale Pagliaro
Dipartimento di Scienze Cliniche e
Biologiche Università di Torino
Ospedale S. Luigi, Regione Gonzole,10
10043 ORBASSANO (TO) Italy
Tel: 39-11 6705430/5450
fax: 39-11 9038639
e-mail: pasquale.pagliaro@unito.it

Abstract

Melusin is a muscle-specific protein which interacts with $\beta 1$ integrin cytoplasmic domain and acts as chaperone protein. Its overexpression induces improved resistance to cardiac overload delaying left ventricle dilation and reducing the occurrence of heart failure. Here we investigated possible protective effect of melusin overexpression against acute ischemia/reperfusion (I/R) injury with or without Postconditioning cardioprotective maneuvers.

Melusin-transgenic (Mel-TG) mice hearts were subjected to 30 minutes global ischemia followed by 60 minutes reperfusion. Interestingly, infarct size (IS) was reduced in Mel-TG mice hearts compared to wildtype (WT) hearts ($40.3 \pm 3.5\%$ Mel-TG vs. $59.5 \pm 3.8\%$ WT hearts; $n = 11$ animals/group; $P < 0.05$). The melusin protective effect was also demonstrated by measuring LDH release, which was 50% lower in Mel-TG compared to WT. Mel-TG hearts had a higher baseline level of AKT, ERK1/2 and GSK3 β phosphorylation, and displayed increased phospho-kinases level after I/R compared to WT mice. Post-ischemic Mel-TG hearts displayed also increased levels of the antiapoptotic factor phospho-BAD. Importantly pharmacological inhibition of PI3K/AKT (Wortmannin) and ERK1/2 (U0126) pathways abrogated the melusin protective effect. Notably, HSP90, a chaperone known to protect heart from I/R injury, showed high levels of expression in the heart of Mel-TG mice suggesting a possible collaboration of this molecule with AKT/ERK/GSK3 β pathways in the melusin-induced protection. Postconditioning, known to activate AKT/ERK/GSK3 β pathways, significantly reduced IS and LDH release in WT hearts, but had no additive protective effects in Mel-TG hearts. These findings implicate melusin as an enhancer of AKT and ERK pathways and as a novel player in cardioprotection from I/R injury.

Key words: melusin; I/R injury; chaperone; Hsp90; AKT; ERK

Introduction

In the heart, ischemia followed by reperfusion (ischemia/reperfusion, I/R) induces all form of cell death, including apoptosis [39, 63], which can be inhibited by pharmacological and genetic approaches resulting in a smaller infarct size [11, 30]. Limitation of myocardial injury after I/R depends on critical adaptive responses, some of which involve the heat-shock proteins (HSPs) which acts as molecular chaperones [37, 46]. In fact, HSP gene expression is induced by virtually any kind of adaptive stress conditioning which reduces the damage of subsequent ischemic insult. The prototype of this phenomenon is known as *preconditioning* (PreC), which provides evidence for adaptive responses to stress by enhancing the cell tolerance to subsequent ischemia [7, 42, 58]. PreC can be achieved in the heart with a variety of stress responses [2, 19, 28, 43, 45], including hypothermia [53] and hyperthermia, which of course induces HSPs [33, 34, 46]. In fact, in PreC two temporary distinct protective windows have been described: a first one, which provides protection against myocardial infarction within 2–3 h after preconditioning stimuli, and a second window of protection, occurring at 24–72 h after PreC stimuli, which is characterized by a unique gene expression profile, including HSPs induction [7, 47]. The introduction of *postconditioning* (PostC) as an intervention which can be applied at the time of myocardial reperfusion, has redirected the attention to the early phase of *reperfusion* as a target for cardioprotection [40, 48, 50, 66, 71]. Studying the signaling moieties implicated in PostC *versus* PreC revealed a common cardioprotective pathway, including, among others, protein kinases such as AKT, ERK1/2 and GSK3 β . These kinases are recruited at time of myocardial reperfusion and are collectively called “reperfusion injury salvage kinase” (RISK)-pathway, which may also recruit anti-apoptotic mechanistic pathways, including the phosphorylation of Bcl-2-associated death promoter (BAD) [13, 24]. The similarity of pathways between pre and postconditioning is in line with the observation that the two protections are not additive [23].

A second important cardioprotective element, common to PreC and PostC, is represented by chaperone proteins, also known as HSPs [12, 18, 41, 63]. We have previously identified melusin as a new muscle-specific chaperone protein binding to the cytoplasmic domain of β 1 integrin and acting as mechanical stretch sensor of the cytoskeleton [8]. Analysis of signaling pathways in transgenic mice indicated that melusin overexpression induced increased phosphorylation of AKT, GSK3 β and ERK1/2 at basal level and after pressure overload [15]. Moreover, AKT and GSK3 β were under-phosphorylated in response to pressure overload in melusin null mice [8].

Therefore, since melusin overexpression enhances the phosphorylation of some RISK pathway components in *non-ischemic* conditions and since melusin has been shown to act as a co-chaperone of HSP90 [57], we hypothesized that melusin overexpression can trigger adaptive responses enhancing the heart tolerance to I/R challenging. To verify this hypothesis, in the present study, we analyzed the myocardial injury in melusin overexpressing and littermate wildtype mice after I/R insult. Moreover, the implication of the AKT and ERK1/2 signaling pathways was analyzed. Finally, the possibility of an additive protective effect by postconditioning was studied in the Mel-TG model.

Methods

Animals

Male FVB non-transgenic (wildtype, WT) and FVB transgenic littermate mice overexpressing melusin (Mel-TG) [15] received humane care in compliance with Italian law (DL-116, Jan. 27, 1992) and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All efforts were made to minimize suffering.

Perfusion technique

Male WT and transgenic littermate FVB mice (Mel-TG) [15] weighing between 25-35 g (10-15 weeks old) were given 500 U heparin and anesthetized with sodium pentothal (50 mg/kg) by intraperitoneal injections before being culled by cervical dislocation [55, 56]. Hearts were rapidly excised and perfused retrogradely at 80 mmHg by the Langendorff technique with Krebs–Henseleit bicarbonate buffer containing (mM) NaCl 118, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.25, and Glucose 11. The buffer was gassed with 95% O₂:5% CO₂. The temperature of the perfusion system was maintained at 37°C. The perfusate flowing out of the heart was collected and measured. Collected coronary effluent was used for measurement of *lactate dehydrogenase* (LDH) release (see below) and the coronary flow rate was determined by the amount of perfusate measured in a specific time period.

At the end of perfusion period, hearts were divided in two parts by a transverse section (perpendicular to the long axis); while the apical part (less than 1/3 of ventricular mass) was frozen rapidly in liquid nitrogen and stored at -80°C and subsequently used for Western blot analysis, the other part was used for infarct size assessment (see below).

Experimental protocol

I/R groups (Fig 1)

Group 1 (WT_I/R, n= 11): in order to have a reference group, hearts were harvested from the WT animals and allowed to stabilize for 30-min. After a 30-min stabilization period, hearts were subjected to a protocol of I/R, which consisted in 30-min of global no-flow, normothermic ischemia and a period of 60-min of reperfusion [4].

Group 2 (WT_PostC, n= 12): after the 30-min ischemia, the WT hearts immediately underwent a protocol of PostC (i.e., 5 cycles of 10-s reperfusion and ischemia) [49, 51].

Group 3 (Mel-TG_I/R, n= 11): hearts were harvested from the transgenic animals (Mel-TG) and, as above, were perfused for a 30-min stabilization period, then global normothermic ischemia was applied by eliminating flow for 30-min, which was followed by 60-min reperfusion.

Group 4 (Mel-TG_PostC, n= 12): after the 30-min ischemia, the Mel-TG hearts immediately underwent a protocol of PostC (i.e., 5 cycles of 10-s reperfusion and ischemia).

Group 5 (Mel-TG_I/R+WM; n= 5): these hearts were treated with 0.1 μ M of the PI3K inhibitor *Wortmannin* (WM) 5-min before ischemia and during the first 20-min of 60-min reperfusion.

Group 6 (Mel-TG_I/R+U0126; n= 5): these hearts were treated with 50 μ M of the MEK1/2 inhibitor *U0126* five min before ischemia and during the first 20-min of 60-min reperfusion.

Groups 7-8 Sham groups (WT_SHAM, n= 3 and Mel-TG_SHAM, n= 3) consisted of hearts which after stabilization underwent 90-min buffer-perfusion only.

The dose of inhibitors WM (0.1 μ M, Sigma, St. Louis, MO) and U0126 (50 μ M, LC Laboratories, Woburn, MA) and the concentration of vehicle (DMSO; Sigma, St. Louis, MO) in which the inhibitors were dissolved were based on previous studies of myocardial I/R injury [10, 36, 49, 51, 52, 70]. Nevertheless, the two inhibitors were tested in hearts of WT animals (n= 3 for each condition) and the maximal DMSO concentration (43 μ l/100ml) was tested in hearts of both strains (n= 3 for each condition).

Assessment of myocardial injury

Infarct size assessment

Infarct areas were assessed at the end of the experiments with the nitro-blue-tetrazolium (NBT) technique in a blinded fashion, as previously described [51]. In brief, immediately after reperfusion, hearts were removed from the perfusion apparatus and, after removing and freezing the apical part (see above), the basal part of the ventricles was dissected by transverse sections into two-three slices.

Following 20-min of incubation at 37°C in 0.1% solution NBT (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer, who was unaware of the protocols. Since the ischemia was global and since we analyzed only the basal part of the ventricles the necrotic mass was expressed as a percentage of the analyzed ischemic tissue.

Lactate dehydrogenase release

Besides infarct size, myocardial injury was also assessed by measuring LDH release. The perfusion effluent was collected for 5-min immediately before ischemia and for the entire reperfusion period. LDH released from the heart was determined by spectrophotometric analysis at 340 nm, using a classic procedure [6, 49].

Western blotting groups

Frozen samples of the four I/R groups (Groups 1, 3, 5 and 6) and samples from other two additional Sham (non-ischemic) groups (Groups 7-8) were used for Western blot analysis. For Western blotting, hearts were lysed in Tris-buffered saline with 1% Triton X-100, plus Roche complete protease inhibitor cocktail containing (mM) NaF 10, PMSF 1 and Na₃VO₄ 1. Protein extracts were clarified with three sequential centrifugations for 20 minutes at 20,000 *g*, at 4°C [55, 56]. Western blot band quantifications were performed with Quantity One software (Bio-Rad).

Antibodies against the following proteins were used: AKT (#9271)(1:1000, Cell Signaling), Phospho-AKT (#9272)(1:1000, Cell Signaling), GSK3β (#610202)(1:1000, BD Transduction Laboratories), Phospho-GSK3β (#9336S)(1:1000, Cell Signaling), BAD (#9292)(1:1000, Cell Signaling), Phospho-BAD (#5284S)(1:1000, Cell Signaling), HSP90 (#610419)(1:1000, BD Transduction Laboratories), GAPDH

(MAB374)(1:1000, Millipore[C1]), Phospho-p70 S6 Kinase (#9206)(1:1000, Cell Signaling), p70 S6 Kinase α (#sc-230)(1:1000, Santa Cruz Biotechnology), ERK1/2 (#sc-93)(1:1000, Santa Cruz) and Phospho-ERK1/2 (#9101)(1:1000, Cell Signaling).

Statistical analysis

All values are expressed as mean \pm SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post test. For all analyses, a minimum value of $P < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software version 4.0).

Results

Melusin overexpression protects against myocardial ischemia/reperfusion injury.

Infarct size, after 60-min of reperfusion increased as the duration of ischemia increased from 25- to 45-min in both heart of wild type (WT) and of melusin transgenic mice (Mel-TG). A period of 30-min of ischemia was determined to be optimal for the comparison of myocardial damage studies. While infarct size was $59.5 \pm 3.8\%$ of risk area in WT hearts subjected to 30-min ischemia and 60-min reperfusion (WT_I/R), it was significantly lower, $40.3 \pm 3.5\%$ in Mel-TG hearts subjected to the same protocol (Mel-TG_I/R) ($P < 0.05$ vs. WT_I/R group) (Fig. 2A).

Moreover, total LDH release after 30-min of global ischemia and 60-min of reperfusion was almost double in WT compared to Mel-TG hearts (431.6 ± 51.1 mU/mg in the WT_I/R and 177.9 ± 19.5 U/mg ww Mel_I/R groups ($P < 0.01$) (Fig. 2B).

We assessed whether the protective effect of melusin overexpression can be further modified by postconditioning maneuvers. As shown in Fig. 2, while the postconditioning maneuvers reduced

significantly the infarct size and LDH release in WT hearts (infarct size $37\pm4\%$ of risk area, $P < 0.01$ vs. WT_I/R group; LDH release 172 ± 24 mU/mg ww; $P < 0.01$ vs. WT_I/R group), these maneuvers had not additive effects on reducing the I/R injury observed in the Mel-TG hearts (infarct size $35\pm3\%$ of risk area, $P =$ not significant (NS) vs. Mel-TG_I/R; LDH release 175 ± 41 mU/mg ww; $P =$ NS vs. Mel-TG_I/R; $P < 0.01$ vs. WT_I/R).

Melusin Protection depends on activation of PI3K/AKT and ERK1/2 pathways

To assess possible mechanisms involved in the melusin induced protection, major signaling pathways known to be regulated by melusin were investigated by Western blot analysis of samples collected from the same hearts used to determine cardiac injury and in the two Sham groups described in the Method section. As shown in Fig. 3, phosphorylation of ERK1/2 (WT_Shram = 0.350 ± 0.063 , Mel-TG_Shram = 1.825 ± 0.014 , $P < 0.001$; WT_I/R = 0.432 ± 0.168 ; Mel-TG_I/R = 1.845 ± 0.182 $P < 0.001$) and AKT (WT_Shram = 0.457 ± 0.032 , Mel-TG_Shram = 2.210 ± 0.034 , $P < 0.05$; WT_I/R = 0.232 ± 0.048 ; Mel-TG_I/R = 1.59 ± 0.585 $P > 0.05$) was potentiated in Mel-TG hearts compared to WT both in basal condition and after I/R. In addition, GSK3 β , a kinase down-stream of AKT, was also more strongly phosphorylated in Mel-TG hearts compared to WT both in basal condition and after I/R (WT_Shram = 1.390 ± 0.016 , Mel-TG_Shram = 2.130 ± 0.043 , $P < 0.05$; WT_I/R = 0.395 ± 0.099 ; Mel-TG_I/R = 1.41 ± 0.383 $P < 0.05$). Moreover, the pro-apoptotic BAD protein was significantly more phosphorylated in Mel-TG hearts subjected to I/R compared to WT (WT_I/R = 0.825 ± 0.207 , Mel-TG_I/R = 1.805 ± 0.192 , $P < 0.05$).

To demonstrate a causal role of increased AKT and ERK1/2 phosphorylation in melusin dependent protection, hearts were treated with the PI3K inhibitor *Wortmannin* (Mel-TG_I/R+WM) and the MEK1/2 inhibitor *U0126* (Mel-TG_I/R+U0126). As shown in Figure 4A, WM treatment resulted in a strong inhibition of AKT phosphorylation as well as of the downstream kinase GSK3 β . Infarct size of Mel-

TG hearts treated with PI3K inhibitor, was significantly higher ($P < 0.01$) with respect to the Mel-TG_I/R group (Figure 4C) and not significantly different from the WT_I/R group ($67.2 \pm 3.9\%$ and $66.4 \pm 8.3\%$, respectively). Accordingly, in Mel-TG_I/R+WM hearts total LDH release was higher ($P < 0.05$) with respect to Mel-TG_I/R group (Figure 4D) and not significantly different from the WT_I/R group (416.5 ± 92.7 U/g ww and 396.3 ± 66.0 U/g ww, respectively).

Similarly MEK 1/2 inhibitor U0126 strongly blunted the phosphorylation of ERK1/2 as well as of the downstream kinase p70S6K (Fig. 4B), abolished the protection in the Mel-TG heart, in fact the infarct size and LDH total release were significantly higher with respect to Mel-TG_I/R group ($68 \pm 4\%$ and 440 ± 83 U/g ww, respectively, $P < 0.001$ respect to Mel-TG_I/R) (Fig 4C, D).

We confirmed in 6 additional WT hearts that the two inhibitors did not exacerbate I/R injury in this strain (Infarct size $61 \pm 3\%$ and $60 \pm 3\%$ and LDH release 446 ± 87 U/g ww and 372 ± 63 U/g ww for WN and U0126, respectively; $P = \text{NS vs. WT_IR}$). These data are in agreement with those reported on previous studies of myocardial I/R injury [10, 52, 70].

Finally, we confirmed in 3 WT hearts that DMSO at the used concentrations had not effect on I/R injury (Infarct size $57 \pm 2\%$ and LDH 375 ± 30 mU/mg, $P = \text{NS vs. WT_I/R}$) [5, 10]. We also showed in 3 Mel-TG hearts that DMSO had no effect on damage (Infarct size $37 \pm 1\%$ and LDH 215 ± 25 U/mg ww, $P = \text{NS vs. Mel_I/R}$).

Melusin overexpression induces HSP90 upregulation

Heat shock proteins are known to protect heart from I/R injury [3, 9, 54, 69]. Given that melusin binds to HSP90 and is co-expressed with HSPs [57], we evaluated the expression of HSP90, an ATP-dependent chaperone known to have a cardio-protective role in I/R [35]. Interestingly, as shown in Figure 3, expression of HSP90 was strongly increased in Mel-TG hearts in basal conditions (WT_Sham =

0.170±0.034, Mel-TG_Shram = 2.230±0.380, $P < 0.01$). Moreover, in Mel-TG mice, HSP90 expression remained higher after I/R (WT_I/R = 0.350±0.063; Mel-TG_I/R = 1.455±0.380 $P > 0.05$).

Discussion

Here we demonstrate, for the first time, that the overexpression of the muscle specific small molecular chaperone *melusin* confers resistance to I/R injury to the heart. Melusin over expression is characterized by increased phosphorylation of AKT/ERK/GSK3 β kinases and overexpression of HSP90. This phenotype represents a protective status relevant for heart tolerance against I/R injury, as shown by the fact that the pharmacological inhibition of PI3K/AKT and MEK/ERK pathways blunted the cardioprotection observed in melusin transgenic mice. Although the involvement of RISK pathway may be species-specific [61], the importance of the PI3K/AKT/ERK pathways in protection from I/R damage has been convincingly demonstrated by a number of different laboratories [24, 27, 38, 48, 60]. Here we demonstrated a key role of the muscle specific protein melusin to regulate these pathways in the context of ischemia and reperfusion. This role is further highlighted by a recent report describing that melusin expression increases after a permanent coronary ligation [22] and that this increase improves cardiac remodeling in a mouse model of myocardial infarction [65].

In our study, due to development of necrosis in late reperfusion (60 min), we could have missed the peak of phosphorylation. In fact, in conditioned hearts maximal AKT and ERK1/2 phosphorylation were observed at 15 min of reperfusion [26]. Moreover, a role may be also played by the dynamic of development of myocardial necrosis and the loss of phospho-AKT and phospho-ERK immunoreactivity, and, specifically, by the different way of tissue sampling [14]. Nevertheless, in our Mel-TG model levels of AKT and ERK1/2 phosphorylation were higher than WT in non-ischemic conditions (Sham). Such higher levels were not reduced after I/R in Mel-TG hearts despite the development of necrosis, thus

supporting an increased process of phosphorylation in post-ischemic phase in surviving cells of melusin transgenic hearts.

The ability of melusin to sustain both the AKT and ERK1/2 pathways is likely based on its function in promoting signalosome complex assembly [20]. Notably, we have previously demonstrated the ability of melusin to bind p85 regulatory subunit of PI3K α [68]. Given that PI3K α is a key upstream regulator of AKT, it can be hypothesized that melusin modulate AKT activation by regulating PI3K α activity. In addition, Melusin also organizes a signalosome complex regulating the ERK1/2 pathway [20]. We have shown, in fact, that melusin interacts with c-Raf, MEK1/2 and ERK1/2 MAP Kinases, as well as with their scaffold molecule IQGAP-1 [55]. An additional key element also present in the complex is the focal adhesion kinase FAK. Biomechanical stress leads to activation of FAK, which in turns triggers the MAPK cascade leading to ERK1/2 activation.

Both the AKT and ERK1/2 pathways impact importantly on cardiomyocyte survival. In fact, AKT, via GSK3 β , plays an important role in the regulation of the mitochondrial permeability transition pore, thus, preventing cardiomyocyte necrosis. At the same time, via BAD phosphorylation it protects cell from apoptosis, a major cause of tissue damage in reperfusion injury [1, 17, 21, 39, 71]. In addition ERK signaling is well known to promote cell survival in different cell types including cardiomyocytes [44, 55, 59]. The MEK/ERK cascade: from signaling specificity to diverse functions [64]. Moreover, a positive crosstalk of ERK and AKT in promoting cells survival has also been reported [16].

An additional component possibly involved in melusin-dependent AKT and ERK activation is the chaperone protein HSP90. In fact, melusin, by binding to HSP90 [32, 57], acts as co-chaperone and likely directs HSP90 toward the signaling substrates, thus, protecting them from dephosphorylation and sustaining their activation state. In this context the up-regulation of HSP90 expression observed in melusin transgenic hearts could represent an important and interesting crosstalk between chaperone

and RISK signaling pathways. However, the present study was not designed to ascertain this crosstalk, which deserves future studies.

The protective effect against reperfusion injury can also be achieved by postconditioning maneuvers [13, 23-25, 27, 36, 48-51, 62, 66, 67, 71]. Our results indicated that a PostC protocol, protective in WT hearts, does not add any further protection in Mel-TG hearts, thus suggesting that the pathway(s) enhanced by melusin is similar to that triggered by PostC.

Our findings suggest potential therapeutic approaches involving the over-expression of melusin. The translation to humans might be obtained either by viral vectors to deliver exogenous melusin genes or by pharmacological treatment capable to increase the expression of the endogenous melusin gene in patients with an high risk profile for heart ischemia. However, the translation of findings on cardioprotection from rodents to humans cannot be taken for granted [29] and analysis in larger mammals, such as the pigs, may be necessary to support translational application [31].

In conclusion, here we present first evidences that melusin overexpression has an important protective effect against ischemia/reperfusion injury *via* its ability to boost RISK pathway signaling and by increasing HSP90 chaperone protein expression.

Acknowledgments

This work was supported by funding from Telethon grant GGP12047 to GT, MIUR Prin 2010RNXM9C_002 to GT; MIUR PRIN 2010J8RYS7_007 to MB and ex-60% to PP.

Conflict of interest: Mara Brancaccio and Guido Tarone are scientific cofounders and consultants for Target Heart Biotec, a company that develops melusin recombinant protein as a drug to counteract heart failure.

References

1. Baines CP (2009) The mitochondrial permeability transition pore and ischemia-reperfusion injury. *Basic Res Cardiol* 104:181-188 doi:10.1007/s00395-009-0004-8
2. Baker JE (2004) Oxidative stress and adaptation of the infant heart to hypoxia and ischemia. *Antioxid Redox Signal* 6:423-429 doi:10.1089/152308604322899495
3. Basso AD, Solit DB, Chiosis G, Giri B, Tschlis P, Rosen N (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem* 277:39858-39866 doi: 10.1074/jbc.M206322200
4. Bell RM, Mocanu MM, Yellon DM (2011) Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *J Mol Cell Cardiol* 50:940-950 doi: 10.1016/j.yjmcc.2011.02.018
5. Bencsik P, Pálóczi J, Kocsis GF, Pipis J, Beleczi I, Varga ZV, Csonka C, Görbe A, Csont T, Ferdinandy P (2014) Moderate inhibition of myocardial matrix metalloproteinase-2 by ilomastat is cardioprotective. *Pharmacol Res* 80:36-42 doi: 10.1016/j.phrs.2013.12.007
6. Bergmeyer HU, Bernt E (1974) Methods of Enzymatic Analysis. In: Bergmeyer HU (ed) Verlag Chemie, Weinheim, Germany, pp 607–612
7. Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, Jadoon AK (1997) Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res* 81:42-52 doi: 10.1161/01.RES.81.1.42
8. Brancaccio M, Fratta L, Notte A, Hirsch E, Poulet R, Guazzone S, De Acetis M, Vecchione C, Marino G, Altruda F, Silengo L, Tarone G, Lembo G (2003) Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. *Nat Med* 9:68-75 doi:10.1038/nm805
9. Budas GR, Churchill EN, Disatnik MH, Sun L, Mochly-Rosen D (2010) Mitochondrial import of PKCepsilon is mediated by HSP90: a role in cardioprotection from ischaemia and reperfusion injury. *Cardiovasc Res* 88:83-92 doi:10.1093/cvr/cvq154
10. Bulhak AA, Jung C, Ostenson CG, Lundberg JO, Sjöquist PO, Pernow J (2009) PPAR-alpha activation protects the type 2 diabetic myocardium against ischemia-reperfusion injury: involvement of the PI3-Kinase/Akt and NO pathway. *Am J Physiol Heart Circ Physiol* 296:H719-H727 doi: 10.1152/ajpheart.00394.2008
11. Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH (2001) Over-expression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *Am J Physiol* 280: H2313-H2320
12. Choudhury S, Bae S, Ke Q, Lee JY, Kim J, Kang PM (2011) Mitochondria to nucleus translocation of AIF in mice lacking Hsp70 during ischemia/reperfusion. *Basic Res Cardiol* 106:397-407 doi: 10.1007/s00395-011-0164-1
13. Cohen MV, Downey JM (2011) Ischemic postconditioning: from receptor to end-effector. *Antioxid Redox Signal* 14:821-831 doi: 10.1089/ars.2010.3318
14. Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K (2005) 'Postconditioning' via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK 1/2. *Am J Physiol Heart Circ Physiol* 289:H1618–H1626 doi:10.1152/ajpheart.00055.2005
15. De Acetis M, Notte A, Accornero F, Selvetella G, Brancaccio M, Vecchione C, Sbroggiò M, Collino F, Pacchioni B, Lanfranchi G, Aretini A, Ferretti R, Maffei A, Altruda F, Silengo L, Tarone G, Lembo

- G (2005) Cardiac overexpression of melusin protects from dilated cardiomyopathy due to long-standing pressure overload. *Circ Res* 96:1087-1094 doi: 10.1161/01.RES.0000168028.36081.e0
16. Dent P (2014) Crosstalk between ERK, AKT, and cell survival. *Cancer Biol Ther* 15:245-246 doi: 10.4161/cbt.27541
 17. Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, Lips DJ, Doevendans PA (2004) Role of apoptosis in reperfusion injury. *Cardiovasc Res* 61: 414-426 doi: 10.1016/j.cardiores.2003.12.023
 18. Efthymiou CA, Mocanu MM, de Bellerocche J, Wells DJ, Latchmann DS, Yellon DM (2004) Heat shock protein 27 protects the heart against myocardial infarction. *Basic Res Cardiol* 99:392-394 doi: 10.1007/s00395-004-0483-6
 19. Engelman DT, Watanabe M, Engelman RM, Rousou JA, Kisin E, Kagan VE, Maulik N, Das DK (1995) Hypoxic preconditioning preserves antioxidant reserve in the working rat heart. *Cardiovasc Res* 29:133-140 doi: 10.1016/S0008-6363(96)88558-0
 20. Ferretti R, Sbroggiò M, Di Savino A, Fusella F, Bertero A, Michowski W, Tarone G, Brancaccio M (2011) Morgana and melusin: two fairies chaperoning signal transduction. *Cell Cycle* 10:3678-3683 doi: 10.4161/cc.10.21.18202
 21. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 94: 1621–1628 doi:10.1172/JCI117504
 22. Gu R, Zheng D, Bai J, Xie J, Dai Q, Xu B (2012) Altered melusin pathways involved in cardiac remodeling following acute myocardial infarction. *Cardiovasc Pathol* 21:105-111 doi: 10.1016/j.carpath.2011.03.002
 23. Halkos ME, Kerendi F, Corvera JS, Wang NP, Kin H, Payne CS, Sun HY, Guyton RA, Vinten-Johansen J, Zhao ZQ (2004) Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. *Ann Thorac Surg* 78:961-969; discussion 969 doi:10.1016/j.athoracsur.2004.03.033
 24. Hausenloy DJ, Lecour S, Yellon DM (2011) Reperfusion injury salvage kinase and survivor activating factor enhancement pro-survival signaling pathways in ischemic postconditioning: two sides of the same coin. *Antioxid Redox Signal* 14:893-907 doi: 10.1089/ars.2010.3360
 25. Hausenloy DJ, Ong SB, Yellon DM (2009) The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. *Basic Res Cardiol* 104:189-202 doi: 10.1007/s00395-009-0010-x
 26. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM (2005) Ischemic preconditioning protects by activating pro-survival kinases at reperfusion. *Am J Physiol Heart Circ Physiol* 288:H971-H976 doi: 10.1152/ajpheart.00374.2004
 27. Hausenloy DJ, Tsang A, Yellon DM (2005) The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc Med* 15:69-75
 28. Heusch G (2013) Remote conditioning: the future of cardioprotection? *J Cardiovasc Med (Hagerstown)* 14:176-179 doi: 10.2459/JCM.0b013e328358e507
 29. Heusch G (2013) Cardioprotection: chances and challenges of its translation to the clinic. *Lancet* 381:166-175 doi: 10.1016/S0140-6736(12)60916-7.

30. Heusch G, Boengler K, Schulz R (2008) Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation* 118:1915-1919 doi: 10.1161/CIRCULATIONAHA.108.805242
31. Heusch G, Musiolik J, Gedik N, Skyschally A (2011) Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. *Circ Res* 109:1302-1308 doi: 10.1161/CIRCRESAHA.111.255604.
32. Hong TJ, Kim S, Wi AR, Lee P, Kang M, Jeong JH, Hahn JS (2013) Dynamic nucleotide-dependent interactions of cysteine- and histidine-rich domain (CHORD)-containing Hsp90 cochaperones Chp-1 and melusin with cochaperones PP5 and Sgt1. *J Biol Chem* 288:215-222 doi: 10.1074/jbc.M112.398636
33. Iliodromitis EK, Karavolias GK, Bofilis E, Yellon DM, Kremastinos DT (1999) Enhanced protection of heat shock in myocardial infarction: inhibition of detrimental effect of systemic hyperthermia. *Cardiovasc Drugs Ther* 13:223-231 doi: 10.1023/A:1007796125902
34. Kingma JG, Jr. (1999) Cardiac adaptation to ischemia-reperfusion injury. *Ann N Y Acad Sci* 874:83-99 doi: 10.1111/j.1749-6632.1999.tb09227.x1
35. Kupatt C, Dessy C, Hinkel R, Raake P, Daneau G, Bouzin C, Boekstegers P, Feron O (2004) Heat shock protein 90 transfection reduces ischemia-reperfusion-induced myocardial dysfunction via reciprocal endothelial NO synthase serine 1177 phosphorylation and threonine 495 dephosphorylation. *Arterioscler Thromb Vasc Biol* 24:1435-1441 doi: 10.1161/01.ATV.0000134300.87476.d1
36. Lacerda L, Somers S, Opie LH, Lecour S (2009) Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovasc Res* 84:201-208 doi: 10.1093/cvr/cvp274
37. Lavu M, Gundewar S, Lefer DJ (2011) Gene therapy for ischemic heart disease. *J Mol Cell Cardiol* 50:742-750 doi: 10.1016/j.yjmcc.2010.06.007
38. Liu Y, Yang XM, Iliodromitis EK, Kremastinos DT, Dost T, Cohen MV, Downey JM (2008) Redox signaling at reperfusion is required for protection from ischemic preconditioning but not from a direct PKC activator. *Basic Res Cardiol* 103:54-59 doi: 10.1007/s00395-007-0683-y
39. Logue SE, Gustafsson AB, Samali A, Gottlieb RA (2005) Ischemia/reperfusion injury at the intersection with cell death. *J Mol Cell Cardiol* 38:21-33 doi: 10.1016/j.yjmcc.2004.11.009
40. Luan HF, Zhao ZB, Zhao QH, Zhu P, Xiu MY, Ji Y (2012) Hydrogen sulfide postconditioning protects isolated rat hearts against ischemia and reperfusion injury mediated by the JAK2/STAT3 survival pathway. *Braz J Med Biol Res* 45:898-905 doi.org/10.1590/S0100-879X2012007500090
41. Marais E, Genade S, Salie R, Huisamen B, Maritz S, Moolman JA, Lochner A (2005) The temporal relationship between p38 MAPK and HSP27 activation in ischaemic and pharmacological preconditioning. *Basic Res Cardiol* 100:35-47 doi: 10.1007/s00395-004-0495-7
42. Marber MS, Latchman DS, Walker JM, Yellon DM (1993) Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88:1264-1272 doi: 10.1161/01.CIR.88.3.1264
43. Maulik N, Watanabe M, Engelman DT, Engelman RM, Das DK (1995) Oxidative stress adaptation improves postischemic ventricular recovery. *Mol Cell Biochem* 144:67-74 doi: 10.1007/BF00926742
44. Mavria G, Vercoulen Y, Yeo M, Paterson H, Karasarides M, Marais R, Bird D, Marshall CJ (2006) ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell* 9:33-44 doi.org/10.1016/j.ccr.2005.12.021

45. Murry CE, Jennings RB, Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74:1124-1136 doi: 10.1161/01.CIR.74.5.1124
46. Okubo S, Wildner O, Shah MR, Chelliah JC, Hess ML, Kukreja RC (2001) Gene transfer of heat-shock protein 70 reduces infarct size in vivo after ischemia/reperfusion in the rabbit heart. *Circulation* 103:877-881 doi: 10.1161/01.CIR.103.6.877
47. Pagliaro P, Gattullo D, Rastaldo R, Losano G (2001) Ischemic preconditioning: from the first to the second window of protection. *Life Sci* 69:1-15
48. Pagliaro P, Moro F, Tullio F, Perrelli MG, Penna C (2011) Cardioprotective pathways during reperfusion: focus on redox signaling and other modalities of cell signaling. *Antioxid Redox Signal* 14:833-850 doi: 10.1089/ars.2010.3245
49. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P (2006) Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. *Basic Res Cardiol* 101:168-179 doi: 10.1007/s00395-005-0543-6
50. Penna C, Perrelli MG, Pagliaro P (2013) Mitochondrial pathways, permeability transition pore, and redox signaling in cardioprotection: therapeutic implications. *Antioxid Redox Signal* 18:556-599 doi: 10.1089/ars.2011.4459
51. Penna C, Tullio F, Merlino A, Moro F, Raimondo S, Rastaldo R, Perrelli MG, Mancardi D, Pagliaro P (2009) Postconditioning cardioprotection against infarct size and post-ischemic systolic dysfunction is influenced by gender. *Basic Res Cardiol* 104:390-402 doi: 10.1007/s00395-008-0762-8
52. Philipp S, Critz SD, Cui L, Solodushko V, Cohen MV, Downey JM (2006) Localizing extracellular signal-regulated kinase (ERK) in pharmacological preconditioning's trigger pathway. *Basic Res Cardiol* 101:159-167 DOI 10.1007/s00395-005-0566-z
53. Prasad MR, Liu X, Rousou JA, Engelman RM, Jones R, George A, Das DK (1992) Reduced free radical generation during reperfusion of hypothermically arrested hearts. *Mol Cell Biochem* 111:97-102 doi: 10.1007/BF00229579 1
54. Sato S, Fujita N, Tsuruo T (2000) Modulation of Akt kinase activity by binding to HSP90. *Proc Natl Acad Sci USA* 97:10832-10837 doi: 10.1073/pnas.170276797
55. Sbroggiò M, Bertero A, Velasco S, Fusella F, De Blasio E, Bahou WF, Silengo L, Turco E, Brancaccio M, Tarone G (2011) ERK1/2 activation in heart is controlled by melusin, focal adhesion kinase and the scaffold protein IQGAP1. *J Cell Sci* 124:3515-3524 doi: 10.1242/jcs.091140
56. Sbroggiò M, Carnevale D, Bertero A, Cifelli G, De Blasio E, Mascio G, Hirsch E, Bahou WF, Turco E, Silengo L, Brancaccio M, Lembo G, Tarone G (2011) IQGAP1 regulates ERK1/2 and AKT signalling in the heart and sustains functional remodelling upon pressure overload. *Cardiovasc Res* 91:456-464 doi: 10.1093/cvr/cvr103
57. Sbroggiò M, Ferretti R, Percivalle E, Gutkowska M, Zylicz A, Michowski W, Kuznicki J, Accornero F, Pacchioni B, Lanfranchi G, Hamm J, Turco E, Silengo L, Tarone G, Brancaccio M (2008) The mammalian CHORD-containing protein melusin is a stress response protein interacting with Hsp90 and Sgt1. *FEBS Lett* 582:1788-1794 doi: 10.1016/j.febslet.2008.04.058
58. Schulz R, Rose J, Post H, Heusch G (1995) Involvement of endogenous adenosine in ischaemic preconditioning in swine. *Pflugers Arch* 430:273-282 doi: 10.1007/BF00374659

59. Shaul YD, Seger R (2007) The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim Biophys Acta* 2007 Aug;1773(8):1213-26 doi.org/10.1016/j.bbamcr.2006.10.005
60. Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, Yellon DM (2007) Postconditioning protects human atrial muscle through the activation of the RISK pathway. *Basic Res Cardiol* 102:453-459 doi: 10.1007/s00395-007-0664-1
61. Skyschally A, Van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G (2009) Ischemic postconditioning in pigs: no causal role for RISK activation. *Circ Res* 104:15-18 doi: 10.1161/CIRCRESAHA.108.186429
62. Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G (2009) Ischemic postconditioning: experimental models and protocol algorithms. *Basic Res Cardiol* 104:469-483 doi: 10.1007/s00395-009-0040-4
63. Suzuki K, Sawa Y, Kagisaki K, Taketani S, Ichikawa H, Kaneda Y, Matsuda H (2000) Reduction in myocardial apoptosis associated with overexpression of heat shock protein 70. *Basic Res Cardiol* 95:397-403 doi: 10.1007/s003950070039
64. Tarone G, Sbroggiò M, Brancaccio M (2013) Key role of ERK1/2 molecular scaffolds in heart pathology. *Cell Mol Life Sci* 70:4047-4054 doi: 10.1007/s00018-013-1321-5
65. Unsöld B, Kaul A, Sbroggiò M, Schubert C, Regitz-Zagrosek V, Brancaccio M, Damilano F, Hirsch E, van Bilsen M, Munts C, Sipido K, Bito V, Detre E, Wagner NM, Schäfer K, Seidler T, Vogt J, Neef S, Bleckmann A, Maier LS, Balligand JL, Bouzin C, Clapier RV, Garnier A, Eschenhagen T, El-Armouche A, Knöll R, Tarone G, Hasenfuß G (2013) Melusin protects from cardiac rupture and improves functional remodelling after myocardial infarction. *Cardiovasc Res* doi: 10.1093/cvr/cvt235
66. Vinten-Johansen J, Granfeldt A, Mykytenko J, Undyala VV, Dong Y, Przyklenk K (2011) The multidimensional physiological responses to postconditioning. *Antioxid Redox Signal* 14:791-810 doi: 10.1089/ars.2010.3396
67. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F (2005) Postconditioning-A new link in nature's armor against myocardial ischemia-reperfusion injury. *Basic Res Cardiol* 100:295-310 doi: 10.1007/s00395-005-0539-2
68. Waardenberg AJ, Bernardo BC, Ng DC, Shepherd PR, Cemerlang N, Sbroggiò M, Wells CA, Dalrymple BP, Brancaccio M, Lin RC, McMullen JR (2011) Phosphoinositide 3-kinase (PI3K(p110alpha)) directly regulates key components of the Z-disc and cardiac structure. *J Biol Chem* 286:30837-30846 doi:10.1074/jbc.M111.271684
69. Willis MS, Patterson C (2010) Hold me tight: Role of the heat shock protein family of chaperones in cardiac disease. *Circulation* 122:1740-1751 doi: 10.1161/CIRCULATIONAHA.110.942250
70. Zhang J, Cai H (2010) Netrin-1 prevents ischemia/reperfusion-induced myocardial infarction via a DCC/ERK1/2/eNOS s1177/NO/DCC feed-forward mechanism. *J Mol Cell Cardiol* 48:1060-1070 doi: 10.1016/j.yjmcc.2009.11.020
71. Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J (2000) Reperfusion induces myocardial apoptotic cell death. *Cardiovasc Res* 45: 651-660 doi: 10.1016/S0008-6363(99)00354-5

Figure Legends

Fig. 1 Experimental protocols. Timeline of the eight experimental groups. Experimental groups 2 and 4: vertical lines represent postconditioning protocol at the beginning of reperfusion. Experimental groups 5 and 6: Wortmaninn (0.1 μ M) and U0126 (50 μ M) were used as inhibitors of PI3K and MEK1/2 respectively. Inhibitors were infused 5 min before and 20 min after ischemia. Arrows indicate the sample collection for Western blot analysis. For acronyms see the text.

Fig. 2 Infarct size and LDH release. Panel A: infarct size (IS) expressed as percentage of the ischemic tissue (IT) resulted smaller in Mel-TG_I/R compared with WT_I/R mice. Panel B: the increase in LDH concentrations during reperfusion was lower in Mel-TG_I/R than in WT_I/R mice. While PostC reduced IS and LDH release in WT heart, it did not add any further protection in Mel-TG model. * $P < 0.05$; ** $P < 0.01$.

Fig. 3 Melusin overexpression induces AKT pathway activation and HSP90 upregulation. Panel A: Western blot analysis of tissue extracts from hearts subjected to ischemia reperfusion or from control hearts. GAPDH was used as loading control. Representative Western blot results are shown. Panel B: Densitometric quantification of Western blot bands. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Fig. 4 Inhibition of either AKT or ERK1/2 phosphorylation blocks protection to ischemia reperfusion induced by melusin overexpression. Western blot analysis of phosphorylated AKT (P-AKT), GSK3 β (P-GSK3 β) (panel A), and phosphorylated ERK (P-ERK) and p70S6K (P-p70S6K) (panel B) on heart extracts from melusin overexpressing mice subjected to ischemia/reperfusion (Mel-TG_I/R) in the presence of

either PI3K inhibitor Wortmannin (WM) or MEK inhibitor U0126. Wild type hearts subjected to ischemia/reperfusion were used for comparative purpose (WT_I/R). The histogram shows the densitometric quantification of Western blot bands. Representative Western blot results are shown. Panel C: infarct size (IS) expressed as percentage of ischemic tissue (IT) resulted larger in Mel-TG treated with Wortmannin or U0126 compared to untreated Mel-TG mice. Panel D: the increase in LDH concentrations during reperfusion was higher in Mel-TG_I/R treated with Wortmannin or U0126 compared to untreated Mel-TG mice. * $P < 0.05$.

Figure 1

Experimental protocols

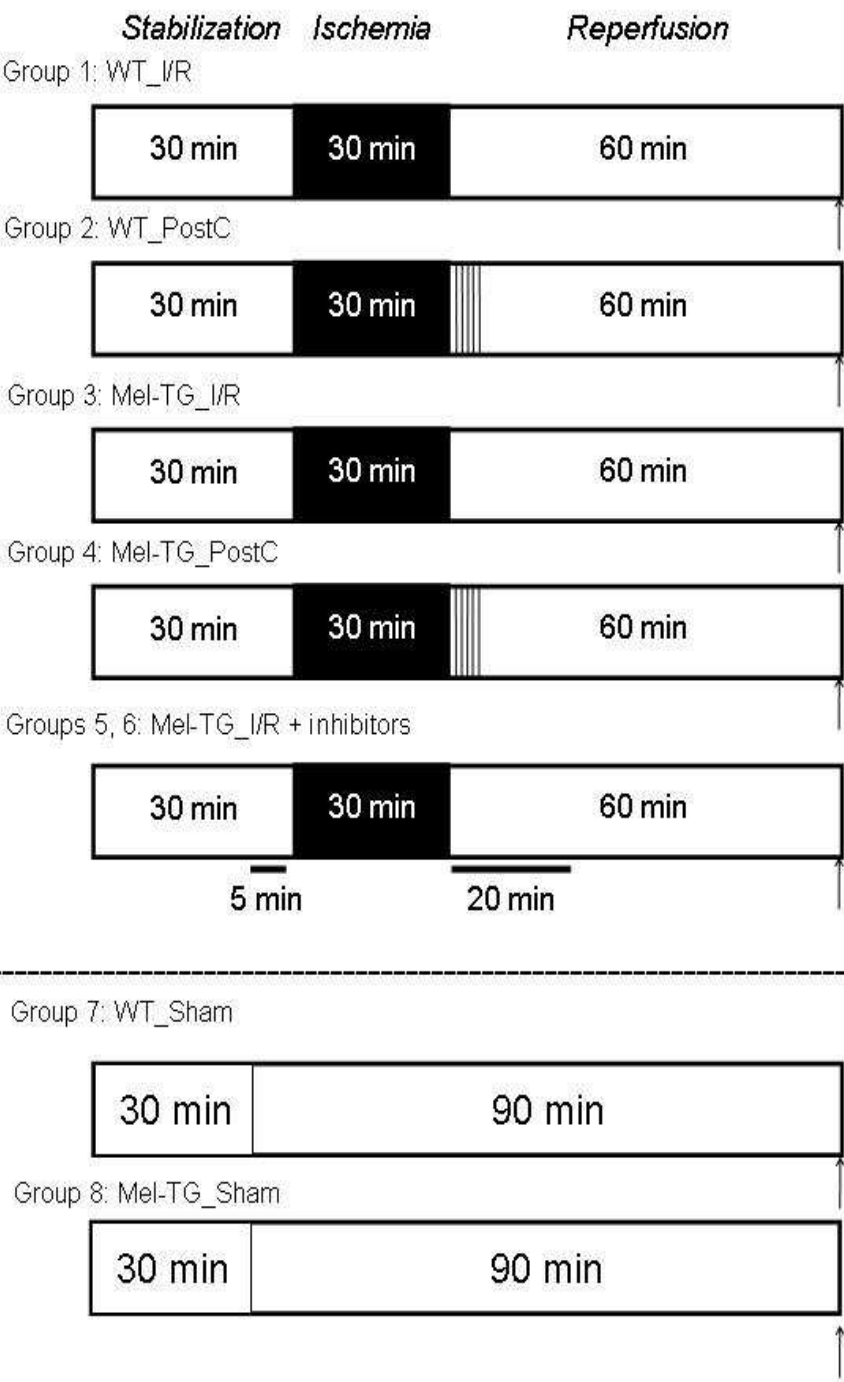
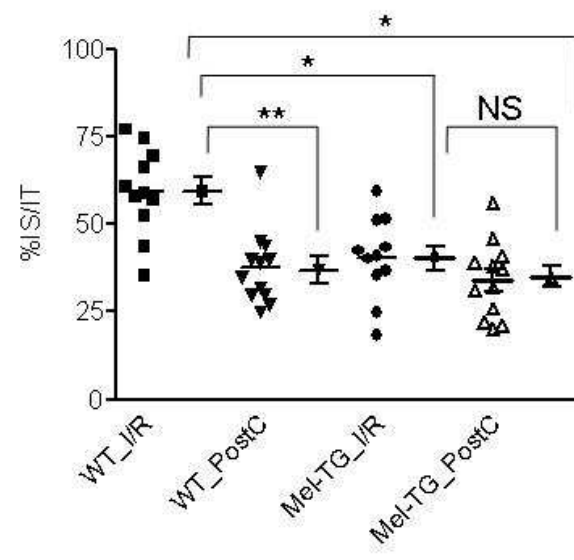


Figure 2

A



B

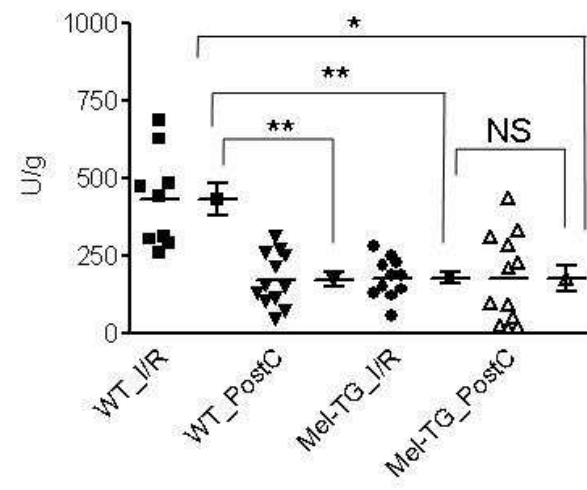


Figure 3

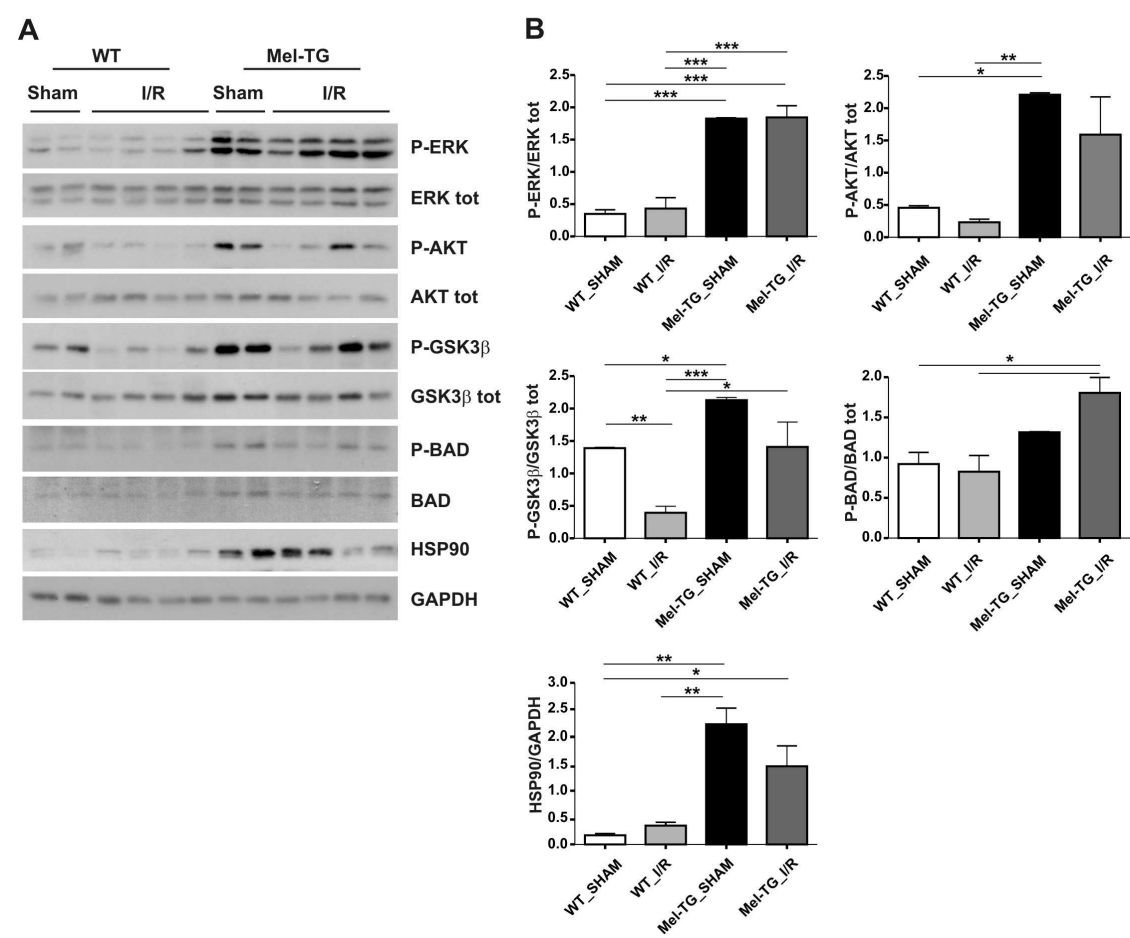


Figure 4

